Sanguansermsri, P., Nobbs, A. H., Jenkinson, H. F., & Surarit, R. (2018). Interspecies dynamics among bacteria associated with canine periodontal disease. *Molecular Oral Microbiology*, *33*(1), 59-67. https://doi.org/10.1111/omi.12199

Molecular Oral Microbiology

Interspecies dynamics among bacteria associated with canine periodontal disease

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Keywords: *Porphyromonas gulae*, *Parvimonas micra*, Biofilms, Coaggregation, Protease

SUMMARY

The etiology and pathogenic mechanisms associated with canine periodontal disease are less well understood than the disease in humans. In this study we have reconstructed defined consortia biofilms in vitro of microorganisms identified as prevalent in a same-breed cohort of dogs with or without periodontal disease. *Frederiksenia canicola* and *Neisseria canis* were selected as potential early colonizers of salivary pellicle, and *Fusobacterium nucleatum* and *Porphyromonas gulae* were included as high incidence canine oral bacteria. *N. canis* formed a biofilm substratum under aerobic conditions, but was unable to tolerate anaerobic conditions. *Fr. canicola* exhibited synergistic biofilm growth with *P. gulae* under anaerobic conditions, but displayed an antagonistic relationship with *F. nucleatum*. However, strong co-adhesion between *F. nucleatum* and *P. gulae* was able to overcome the inhibitory effects of *Fr. canicola* to facilitate three-species biofilm formation. *Parvimonas micra*, an anaerobic, asaccharolytic Gram-positive coccus found only under disease conditions in vivo, was able to form biofilms in conjunction with *Fr. canicola* and *P. gulae*. Furthermore, the specific proteolytic activities of biofilms containing *Fr. canicola* and *P. gulae* or *F. nucleatum* and *P. gulae* were several-fold increased upon the addition of *Pa. micra*. This suggests that anaerobic cocci such as *Pa. micra* might provide a catalyst for progressive tissue destruction, inflammation, and alveolar bone loss in canine periodontal disease, in keeping with the keystone-pathogen hypothesis.

INTRODUCTION

In humans, periodontitis is a biofilm-induced chronic inflammatory condition characterized by gingival recession and alveolar bone loss. The disease is multifactorial in that a wide range of microorganisms, microbial products, and host immune responses have been implicated [1]. While dental plaque biofilms play a main role in triggering periodontitis, it is the host inflammatory responses that cause destruction of the periodontium (tooth-supporting tissues). The presence of three species of Gram-negative anaerobic bacteria within sub-gingival plaque, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, has been strongly associated with diseased sites [2], although the etiology is much more complex [3]. These invasive bacteria produce high levels of protein-degrading activities which appear to be important for virulence [4-6]. However, the notion that periodontitis is the direct result of these so-named periodontopathogens has been redefined by the keystone-pathogen hypothesis [7]. In the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease, keystone pathogen *P. gingivalis* impairs host defense leading to overgrowth of oral commensal bacteria, thus transforming the normally symbiotic microbiota into a dysbiotic state. Environmental changes induced by the keystone-pathogen then favor proteolytic bacteria and facilitate compositional changes in the biofilm community [8].

Periodontal disease is also a major affliction in dogs affecting approximately 60% of the population [9]. Previous studies revealed similarities in subgingival plaque composition between dogs and humans at the bacterial genus level [10]. However, the canine oral microbiome is widely divergent from that of the human, with at least 350 canine bacterial taxa identified [11] of which only 16.4% are shared with humans. Subgingival plaque collected from non-diseased sites in dogs contained mainly Gram-negative aerobic bacteria of the genera *Moraxella*, *Bergeyella*, *Neisseria* and *Capnocytophaga*, and family *Pasteurellaceae* spp. [12], while in plaque from mild periodontitis there were higher proportions of species from

the class Clostridia. A longitudinal study of sub-gingival plaque community changes associated with development of canine periodontal disease essentially confirmed that aerobic Gram-negative species decreased in proportion as periodontitis developed [13] while *Peptostreptococcaceae* increased. *Porphyromonas cangingivalis*, *Porphyromonas gulae* and other *Porphyromonas* species were present at all sites. These observations tend to infer a community-wide transition into periodontitis and in this respect is broadly consistent with current hypotheses for development of periodontal disease in humans [8].

The primary colonizers of the tooth surface in humans are streptococci, along with actinomyces, neisseriae and veillonellae [14,15]. Secondary colonizers such as *Fusobacterium nucleatum* and *P. gingivalis* benefit from the antecedent community providing new attachment sites and metabolic compatibilities [15,16]. However, streptococci are generally lacking in canine plaque biofilms [12,17] and it would appear that Gram-negative aerobic or facultatively anaerobic bacteria are primary colonizers [17]. In a model of early canine plaque development these organisms might then provide for incorporation into the community of secondary colonizers such as fusobacteria, porphyromonads and peptostreptococci [18]. Although oral microbial community development in humans has been widely investigated [15,19] the interactive processes involved in the development of canine plaque biofilms are much less well understood. Our recent studies with a cohort of same-breed dogs have shown that *Frederiksenia canicola*, within the family *Pasteurellaceae*, was commonly present at non-diseased sites, while bacteria found strictly associated with periodontitis in dogs included *Parvimonas micra*, *Peptostreptococcus canis* and *Porphyromonas crevioricanis* [20]. Organisms located both at non-diseased and diseased sites included *F. nucleatum* and *P. gulae*. In this present study, we have utilized fluorescent *in situ* hybridization (FISH) together with confocal laser scanning microscopy (CLSM) to investigate the potential interactions occurring between specific canine plaque bacteria during early biofilm development.

Knowledge of interspecies relationships will provide new information about oral biofilm initiation and development in dogs, and lead to better understanding of the etiology of periodontitis and improving strategies for disease control and prevention.

METHODS

Bacterial strains and culture conditions

F. nucleatum ATCC 25586, *Neisseria canis* OH217, *Pa. micra* W2856 and *P. gulae* UB1945 were from the University of Bristol Oral Microbiology Culture Collection. *N. canis* and *P. gulae* were isolated from canine subgingival plaque samples, while *F. nucleatum* and *Pa. micra* were human isolates. *Fr. canicola* HPA 21 [21] canine isolate was a gift from P. Kuhnert (University of Bern, Switzerland). *P. crevioricanis* CCUG57307, isolated from the dog oral cavity,was obtained from the University of Göteborg, Sweden Culture Collection. *F. nucleatum, N. canis, Pa. micra* and *P. gulae* were routinely grown on Columbia agar under anaerobic conditions, except for *N. canis* which was cultivated aerobically. *P. crevioricanis* was grown anaerobically on Chocolate agar, and *Fr. canicola* was cultivated on Tryptone Soya agar aerobically, although this species was also able to grow anaerobically. All agar media were supplemented with 5% defibrinated horse blood. Strictly anaerobic bacteria (*F. nucleatum, Pa. micra, P. crevioricanis, P. gulae*) were cultivated at 37ºC in Fastidious Anaerobic Broth containing 5% defibrinated horse blood under N_2:CO_2:H_2 (85:10:5) for 4-7 days. *N. canis* was grown aerobically at 37ºC in Brain Heart Infusion broth supplemented with yeast extract for 24 h, while *Fr. canicola* was grown aerobically at 37ºC in Tryptone Soya broth for 2 d. Growth media for *P. crevioricanis* and *P. gulae* cultures additionally contained hemin (5 μg ml^{-1}) and menadione (1 μg ml^{-1}). The identities of all bacterial strains

cultivated were confirmed by sequencing of 16S rRNA gene products generated by PCR with specific primers listed in Table 1.

Bacterial coaggregation

Bacterial strains were grown in respective media as described above. Cells were harvested by centrifugation at 5,000 ***g*** for 7 min at 4°C and suspended in coaggregation buffer (1 mM Tris-HCl pH 8, 150 mM NaCl, 0.1 mM $CaCl_2.2H_2O$, 0.1 mM $MgCl_2$ and 0.02% NaN_3). The optical density at 600 nm of each suspension was adjusted to OD_{600} =1 (estimated 2-7 x 10^8 cells ml^{-1} depending upon bacterial strain). Equal volumes of each bacterial suspension (1 ml) were vortex-mixed for 10 s in a glass tube and then allowed to stand at room temperature for 5 min. The extent of coaggregation was scored, with the aid of a light microscope, from 0-4 as follows: 0 = uniformly turbid and no aggregate suspension of bacteria visible; 1 = very small clumps in a turbid background; 2 = definite clumps of bacteria visible but remaining in a turbid background; 3 = large aggregates with little background; 4 = large aggregates with completely clear supernatant [22]. The experiments were repeated 3 times to confirm scores.

Biofilm development and confocal laser scanning microscopy (CLSM)

Bacterial suspension cultures were centrifuged at 5,000 ***g*** for 7 min, pellets were suspended in canine artificial saliva (CAS) medium (pre-reduced for the anaerobic species) and incubated at 37ºC to OD_{600} = 0.5. CAS contained (per liter): 1 g Lab Lemco Powder, 2 g Yeast extract, 5 g Proteose peptone, 2.5 g Hog gastric mucin (Sigma-Aldrich, St. Louis, MO, USA), 2.34 g NaCl, 1.5 g KCl, 0.1 g $CaCl_2$ and 1.25 ml 40% urea [18]. Plastic dishes (35 mm diameter) with a 14-mm glass panel bottom microwell were incubated with CAS for 2 h at room temperature. The CAS was removed and 2 ml bacterial suspensions in CAS (OD_{600} = 0.5)

were added. The biofilm cultures were then incubated with gentle shaking at 37ºC in air for 24 h (*N. canis* and *Fr. canicola*) or under anaerobic conditions for 2 d (four other species). For multispecies biofilms involving *N. canis* or *Fr. canicola*, the plates were first incubated with bacterial cell suspension in CAS at 37ºC for 24 h in air. The primary suspensions were then removed, secondary anaerobic bacterial cell suspensions in CAS (final volume 2 ml) were added as appropriate, and the plates were incubated anaerobically for 2 d. The suspensions were then aspirated from the plates and the biofilms were subjected to fluorescence *in situ* hybridization analysis (FISH) as described [23] with modifications. Briefly, biofilms formed on the glass panels were fixed with 4% paraformaldehyde for 2 h, washed with phosphate-buffered saline (PBS) and incubated with 0.1 M Tris-HCl pH 7 containing 10 mg lysozyme ml^{-1} and 500 U ml^{-1} mutanolysin for 8 min at 37°C. The biofilms were then incubated in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7, 25% formamide and 0.01% sodium dodecyl sulfate, SDS), containing 5 µg fluorescent-dye-labeled oligonucleotide probes (Table 2) for 150 min at 55°C. Washing buffer (0.15 M NaCl, 20 mM Tris-HCl pH 7, 0.01% SDS) was then added and the samples were incubated for 15 min at 55°C. Biofilms were analyzed by CLSM using a Leica SP5-AOBS confocal microscope attached to a Leica DM I6000 inverted epifluorescence microscope. The images presented are representative from multiple experiments (n = 8)

Protease activity in biofilms

Protease activities of biofilms were measured using Protease Fluorescent detection kit (Sigma-Aldrich). Briefly, bacterial biofilms were grown in duplicate on CAS-coated glass cover slips (19 mm diameter) in 12-well culture dishes under the conditions described above for 2 d. Cover slips were transferred to fresh wells and washed once with 0.5 ml CAS. Incubation buffer (20 mM sodium phosphate pH 7.6 containing 0.15 M NaCl) was added and

a cell scraper was used for removing and harvesting the bacterial cells. Portions were then mixed with FITC-casein substrate and incubated at 37ºC for 24 h in the dark. Trichloroacetic acid (0.6 M) was added and the mixture incubated at 37°C for 30 min in the dark. The suspensions were then centrifuged at 10,000 ***g*** for 10 min at room temperature to sediment undigested substrate. Portions of supernatant were mixed with Tris-HCl pH 8.5 to neutralize, aliquots (0.2 ml) were transferred to 96-dark-well plates, and fluorescence intensities were recorded with excitation at 485 nm and emission wavelength of 535 nm. Standard curves were generated for trypsin (10 U μg^{-1}) in the range 0.5 - 50 ng ml^{-1} and activities of controls and samples were expressed in Units (U) of proteolytic activity (the amount (in mg) of casein released in 24 h at 37ºC). For measuring total protein in the biofilm samples, biofilms were solubilized in 0.1 M NaOH at 95ºC for 10 min. Total protein in the solutions was determined using Bradford Protein assay and protease activities were expressed as U mg^{-1} biofilm protein.

Statistical analysis

Results were evaluated by analysis of variance and the Dunnett multiple-comparison test using GRAPHPAD PRISM, version 6.0 (GraphPad Software, San Diego, CA, USA). All experiments were performed at least twice.

RESULTS

Coaggregation

We have previously identified *Fr. canicola* and *N. canis* as high-incidence bacteria at non-diseased sub-gingival sites, and *P. crevioricanis and Pa. micra* as higher-incidence microorganisms associated with diseased sites. Since coaggregation between bacterial species

plays an integral role temporally and spatially in oral biofilm development [24] we tested the abilities of various selected canine plaque microorganisms to interact with each other. Pairs of the different species were systematically mixed and assayed for coaggregation as described in Methods. *Fr. canicola* coaggregated strongly with *Pa. micra* (Table 3), and less strongly (score = 2) with *N. canis* (after correction for self-aggregation), *F. nucleatum* and *P. gulae*. *N. canis* was the only microorganism to coaggregate with *P. crevioricanis*, and *N. canis* also showed moderate coaggregation with *Pa. micra* and *P. gulae*. *N. canis* exhibited self-aggregation (Table 3) which was taken into account when interpreting the coaggregating pair scores. None of the disease-associated anaerobic bacteria coaggregated with each other (Table 3). These results supported the hypothesis that *Fr. canicola* or *N. canis* might function as early colonizers and provide a substratum for adherence of secondary colonizers such as *F. nucleatum, Pa. micra, P. crevioricanis* or *P. gulae*.

Mono-species biofilms

All of the bacterial species tested were able to form mono-species biofilms on CAS-coated surfaces, with varying efficiencies. *N. canis, F. nucleatum* and *P. gulae* produced robust biofilms with different architectures (Fig. 1). *N. canis* formed patches of dense growth, under aerobic conditions, with appearance suggestive of extracellular polymeric substances (EPS) (Fig. 1B), but did not form a biofilm under anaerobic conditions (not shown). Biofilms of *F. nucleatum* exhibited a relatively even distribution of rod-shaped organisms across the substratum surface (Fig. 1C). *Fr. canicola* produced relatively sparse biofilms (Fig. 1A) while *Pa. micra* and *P. crevioricanis* were the weaker biofilm producers (Fig. 1D, F). *P. gulae* produced a uniform biofilm comprised of small groups of cells (Fig. 1G).

Multi-species biofilms with *Fr. canicola*

On the basis of the coaggregation results, and on the frequency of detection of the various species in vivo [20], we then tested the abilities of specific organism pairs to grow together in biofilms. Unfortunately, no stable biofilms of *N. canis* could be maintained under anaerobic conditions, resulting in considerable detachment of the biofilm from the surface (data not shown). *P. gulae* formed robust biofilms with *Fr. canicola* under anaerobic conditions (Fig. 2A) compared to the respective mono-species biofilms. Although it is stressed that we are not able to quantify accurately the biomass or biovolume from these FISH data, it is possible to conclude that these organisms are able to co-exist in a co-operative manner. On the other hand, while *Fr. canicola* and *F. nucleatum* were able to form dual-species biofilms, the proportion of *F. nucleatum* was much reduced compared to *F. nucleatum* mono-species, suggesting that there was competition for growth between these species, or possibly inhibition of *F. nucleatum* by *Fr. canicola* (Fig. 2B). *F. nucleatum* clearly was able to form dual-species biofilms with *P. gulae* (Fig. 2C). This interactive relationship appeared to overcome at least in part the antagonistic effects of *Fr. canicola* on *F. nucleatum* (Fig. 2B), since three-species biofilms were readily formed with these bacteria (Fig. 2D). Thus *P. gulae* seems to be a key partner microorganism, its strong associations with *Fr. canicola* and with *F. nucleatum* facilitating the development of large, multi-species coaggregates between these three species (Fig. 3).

Multi-species biofilms with Gram-positive cocci

Pa. micra is an anaerobic Gram-positive coccus, the presence of which in sub-gingival plaque was associated with periodontal disease in the dog cohort under study. We examined therefore the ability of this bacterium to form biofilms in combination with early colonizers

i.e. *Fr. canicola,* and with other anaerobic bacteria e.g. *P. gulae*. *Pa. micra* was able to form a weak biofilm with *Fr. canicola,* although there was little evidence that these organisms were involved in co-adherence (Fig. 4A). Upon the addition of *P. gulae,* the interactions between *P. gulae* and *Fr. canicola* were observed as before, and levels of *Pa. micra* appeared to be reduced (Fig. 4B).

We also examined the ability of *P. crevioricanis* to be incorporated into biofilms of *P. gulae* and *Fr. canicola*. However, there was little or no indication that these bacteria would co-associate (data not shown).

Biofilm protease activity

Since protease production has been associated with periodontal tissue destruction, and the proteases of *P. gingivalis* have multiple roles in pathogenesis [25], we investigated the possible links between biofilm formation and protease production. In particular, *P. gulae* has been shown to express a higher specific activity of protease than *P. gingivalis* [26]. Of the six canine bacterial species studied here, *P. gulae* biofilms contained the highest protease (caseinolytic) activity (Fig. 5). *Pa. micra* and *P. crevioricanis* biofilms also had protease activity but approximately 5-fold or more less than *P. gulae*. Specific protease activities in biofilms comprising *P. gulae* + *Fr. canicola* or *P. gulae* + *F. nucleatum* were not significantly different from *P. gulae* mono-species biofilms (Fig. 5). However, as soon as *Pa. micra* was introduced into the biofilms the protease specific activities were significantly elevated (Fig. 5). This effect was specific to *Pa. micra,* and was not seen with *P. crevioricanis* (Fig. 5). Protease activities of biofilms consisting of *Pa. micra* alone with *Fr. canicola* or *F. nucleatum* were very low (Fig. 5). In summary, *Pa. micra* appears to enhance protease activity in biofilms containing *P. gulae* with *F. nucleatum* or *Fr. canicola*. These

observations would be in keeping with the *in vivo* evidence that higher incidence of Gram-positive cocci, in particular *Pa. micra*, in combination with *P. gulae* and *F. nucleatum*, might be associated with periodontal disease pathogenesis in dogs.

DISCUSSION

Information about the composition of microbiomes associated with multiple environments in animals is rapidly increasing, and companion animals are no exception. The canine oral microbiome [11], based upon pooled samples from multiple dogs of different breeds, revealed that the bacterial taxa present were markedly different from humans. Subsequent analyses of sub-gingival plaque microbiomes from single breeds of dogs [13,17] have provided more detailed information for specific cohorts of animals. General conclusions from these studies were that *Neisseria*, *Corynebacterium*, *Bergeyella* and *Moraxella* were most prominent and that reduced proportions of these genera in sub-gingival plaque were associated with periodontal disease. The notion of a shift from Gram-positive bacteria (e.g. *Streptococcus*) to predominantly Gram-negative bacteria (e.g. *Porphyromonas*, *Tannerella*) in humans developing periodontitis [2] is therefore not paralleled. However, there are some similarities, for example the presence of *Neisseria* and *Moraxella* in early plaque, and the high incidence of *Treponema* in periodontal lesions of humans and dogs [2,4,10,11,20]. The microbiome information needs to be translated into functional processes to better understand disease mechanisms, and to do this we have reconstructed some of the in vivo microbial components as in vitro biofilms to investigate interactive events.

The microorganisms that we have utilized in these studies were identified as prevalent species associated with non-diseased or diseased sites in a defined cohort of dogs [20]. There

was obviously a plethora of different species present in these samples, but we identified *Neisseria*, *Enhydrobacter*, *Fusobacterium* and *Porphyromonas* spp. as high prevalence with non-diseased animals. A new finding was the high prevalence of *Frederiksenia* (formerly *Bibersteinia*) which we subsequently incorporated into our in vitro studies. Conversely, we only found *Pa. micra* and *P. crevioricanis* associated with diseased sites. *P. gulae* and *F. nucleatum* were commonly found at all sites. This information therefore formed the basis for our biofilm reconstructions. However, we recognize there are limitations in our studies utilizing individual isolates of bacteria, unavoidable at present. For example there are multiple canine oral taxa for *Fusobacterium*, *Porphyromonas* and *Parvimonas* [11], and so the phenotypic properties of different isolates within genera or species may turn out to be different.

Fr. canicola had the capacity to form biofilms with nearly all of the other species, except *P. crevioricanis*, and the pattern of interactions in development of biofilms mirrored very closely the ability of the same organisms to coaggregate. This suggested that the general ideas of succession of microorganisms in plaque development being associated at least in part with the ability of species to interact physically [27,28] holds true for canine dental plaque formation. On the other hand, *P. gulae* and *F. nucleatum* did not exhibit coaggregation in planktonic phase, but appeared to interact on the substratum surface of dual-species biofilms. This suggests that coaggregation in fluid phase is not necessarily a true indicator as to whether or not two or more species can interact when present upon a surface or within a biofilm. Similar observations have been made with *P. gingivalis* and *Streptococcus gordonii*, which do not interact in conventional coaggregation assays, but are able to co-adhere to a substratum and form metabolically active biofilms [29]. *N. canis* coaggregated with all of the anaerobic bacteria, but we consistently found difficulties in generating mixed-species biofilms of obligately-aerobic *N. canis* with anaerobic bacteria. This is something that will

need to be addressed technically for future studies of this sort. Because of this we focused on *Fr. canicola* (which was able to grow aerobically and to an extent anaerobically) as a potential primary colonizer in the longer-term development of sub-gingival communities.

We were able to generate early-stage biofilms comprising *Fr. canicola, P. gulae* and *F. nucleatum*, which were analogous to the kinds of organisms associated with non-diseased sites. There was evidence for synergy between *Fr. canicola* and *P. gulae*, and competition between *Fr. canicola* and *F. nucleatum*. CAS medium was used as a substitute for canine saliva because of the quantities of saliva that would otherwise be required for the present studies. In preliminary experiments CAS was found to support similar levels of biofilm growth of *Fr. canicola* or *P. gulae* as natural canine saliva (data not shown). The anaerobic bacteria, with the exception of *Fr. canicola*, are asaccharolytic, so their growth would be promoted by the peptide components present in CAS. On the other hand, the degradation of mucin present in CAS might be the major growth and energy source for *Fr. canicola*. It is possible therefore that the proteolytic activity of *P. gulae* and the oxidase and catalase activities [21] of *Fr. canicola* provide for mutual benefit of the dual species biofilm.

Biofilms containing *Pa. micra* or *P. crevioricanis* were presumed as indicative of diseased sites. We found that *Pa. micra*, of the order Clostridiales, was incorporated into biofilms with *Fr. canicola*, but its relative proportions were suppressed upon addition of *P. gulae*. *P. crevioricanis* was more difficult to detect by FISH within similar biofilms. Despite apparent reduction in cell numbers, the striking effect of the presence of *Pa. micra* within three-species biofilms was the increase in protease activity observed. We did not acquire protease activity data for dual-species biofilms of *P. gulae* and *Pa. micra* because all of our communities were reconstructed upon a primary organism (*Fr. canicola, F. nucleatum* or *N. canis*). All *P. gulae* strains examined produce Arg-X and Lys-X proteases, similar to the gingipains of *P. gingivalis* [26]. *P. gulae* was the most proteolytic bacterial species of the six

included here. Although *Pa. micra* also produces protease [30]the biofilm specific activity was at least 5-fold lower than *P. gulae* and was not influenced by the presence of *F. nucleatum* or *Fr. canicola*. These observations therefore identify a mechanism whereby proteolytic activity in *P. gulae*-containing biofilms is enhanced by the presence of *Pa. micra*. The increased protease activities might be the result of more efficient degradation of the substrate by *P. gulae* and *Pa. micra* enzymes working in concert. Alternatively, or in addition, *P. gulae* protease gene expression or protease activity might be enhanced by *Pa. micra* metabolic products. These observations of elevated protease levels might therefore begin to account for the association of these Gram-positive asaccharolytic bacteria with disease, since the *P. gulae* proteases alone promote tissue-destruction and pro-inflammatory cytokine production (Lenzo *et al*., 2016). It is interesting to note that *Filifactor alocis*, another Gram-positive asaccharolytic bacterium, has been implicated in human periodontal disease [31]. It would be important to determine if *F. alocis* could potentially enhance the virulence and immunological characteristics of *P. gingivalis* in a similar manner.

In summary, our studies here begin to translate some of the canine microbiome data into dynamic processes occurring during sub-gingival plaque formation. It seems plausible that in the canine scenario of periodontitis, *P. gulae* might behave as a keystone pathogen [7] in first generating an environment conducive to colonization by *Peptoniphilaceae* family bacteria (e.g. *Pa. micra*). This could then lead to elevation in protease levels, further tissue destruction, and favor asaccharolytic bacteria, setting off a cycle of self-nutrition and sustained periodontal disease.

ACKNOWLEDGEMENTS

We thank Lindsay Dutton and Jane Brittan for excellent technical assistance, Katy Jepson and Alan Leard for their valuable training and help with CLSM conducted in the Wolfson Bioimaging Facility, University of Bristol, and Rich Lamont for helpful discussions. P.S. and R.S. were funded by The Royal Golden Jubilee PhD program from the Thailand Research Fund (Grant number PHD/0057/2554) which is gratefully acknowledged.

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Table 1 Primers utilized to confirm bacterial species identities.

Bacterial species	Forward primer	Reference	Reverse primer	Reference
Fr. canicola	CATGCAAGTCGAACGGT	This study	CAGACTCCAATCCGGACTT	This study
N. canis	AGAGTTTGATCCTGGCTCAG	32	GGGCGGTGTGTACAAG	This study
P. crevioricanis	ACTCCTACGGGAGGCAGCAGT	33	TATTACCGCGGCTGCTGGC	33
F. nucleatum, Pa. micra, P. gulae	AGAGTTTGATCCTGGCTCAG (16S rRNA universal primer F27)	32	ACGGGCGGTGTGTAC (16S rRNA universal primer R1329)	34

Table 2 Sequences of fluorescent-dye-labeled oligonucleotide probes[1] used in FISH

Bacterial species	Probe sequence	Dye 5'-modification	Reference
Fr. canicola	CACATTCACATCTCTGCGAAC	Alexa647	This study
F. nucleatum	CTTGTAGTTCCGCTTACCTC	Alexa555	35
P. crevioricanis	ATTGCAAGTACCCTGCGAATAAG	Alexa647	This study
Pa. micra	TCCAGAGTTCCCACCTCT	Alexa555 or Dy415	36
P. gulae	TGCTTATTCTTACGGTACATTCACAG	Alexa488	This study

[1] synthesized by Eurofins Genomic Services Ltd., Wolverhampton, UK

Table 3 Coaggregation scores[1] between different species of canine oral bacteria.

Coaggregating species	***Fr. canicola***	***F. nucleatum***	***N. canis***	***Pa. micra***	***P. crevioricanis***	***P. gulae***
Fr. canicola	1[2]	2	4	3	1	2
F. nucleatum		0	2	0	0	0
N. canis			2[2]	3	4	3
Pa. micra				0	0	0
P. crevioricanis					0	0
P. gulae						0

[1] Extent of coaggregation was scored visually from 0 (no coaggregation) to 4 (complete coaggregation) as described in Methods. Results are from three individual experiments (n = 3).

[2] Self-aggregation

Legends to Figures

Figure 1 Three dimensional CLSM images of mono-species biofilms formed on canine artificial salivary pellicle after incubation at 37 ^{o}C for 24 h in air (Panels A, B), or 48 h anaerobically (Panels C-F). Biofilms were subjected to FISH analysis as described in Methods with probes listed in Table 2. A, *Fr. canicola*; B, *N. canis*; C, *F. nucleatum*; D, *Pa. micra*; E, *P. gulae*; F, *P. crevioricanis.*

Figure 2 CLSM images of dual- or three-species biofilms of *Fr. canicola*, *F. nucleatum* or *P. gulae.* A, *Fr. canicola* (blue) and *P. gulae* (green); B, *Fr. canicola* (blue) and *F. nucleatum* (red); C, *F. nucleatum* (red) and *P. gulae* (green); D, *Fr. canicola* (blue), *F. nucleatum* (red) and *P. gulae* (green).

Figure 3 Higher-magnification CLSM images of mixed-species biofilms showing intergeneric interactions. Arrows indicate three-way associations between *Fr. canicola* (blue), *F. nucleatum* (red) and *P. gulae* (green).

Figure 4 CLSM images of dual- or three-species biofilms of *Fr. canicola*, *Pa. micra* or *P. gulae*. A, *Fr. canicola* (blue) and *Pa. micra* (red); B, *Fr. canicola* (blue), *Pa. micra* (red) and *P. gulae* (green).

Figure 5 Specific protease activities associated with mono-species or mixed-species biofilms. Cells were harvested from biofilms and assayed for protease and total protein, as described in

Methods, and specific activity expressed as Units mg^{-1}. Error bars represent ±SD from three independent experiments (n = 3), and significant differences (P<0.05) are indicated *. Abbreviations for microorganisms: Fc, *Frederiksenia canicola*; Nc, *Neisseria canis*; Fn, *Fusobacterium nucleatum*; Pm, *Parvimonas micra*; Pcr, *Porphyromonas crevioricanis*; Pg, *Porphyromonas gulae*.